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Molecular Analysis of Mixed Infection With Hepatitis C Virus and Human Immunodeficiency Virus in a Patient Infected Simultaneously

Cinzia Mazza, Massimo Puoti, Antonella Ravaggi, Filippo Castelnuovo, Alberto Albertini, and Elisabetta Cariani

III Laboratory of Clinical Chemistry, Hospital of Brescia and Institute of Chemistry School of Medicine (C.M., A.R., A.A., E.C.); Institute of Infectious Diseases (M.P., F.C.), University of Brescia, Brescia, Italy

A case of simultaneous infection with HIV and HCV characterized by a rapidly progressive clinical course was studied retrospectively over 3.5 years. Molecular analysis indicated interference between HIV and HCV and between HCV subtypes 1a and 1b. An ineffective immune response was suggested by the persistence and sequence conservation of the HCV HVR1 variants isolated during the follow-up.

KEY WORDS: coinfection with HCV and HIV, HCV RNA titre, HIV RNA titre, HCV genotypes, HCV hypervariable region 1 (HVR1)

INTRODUCTION

Hepatitis C virus (HCV) has been identified as the main causative agent of blood-borne non-A, non-B hepatitis [Choo et al., 1989]. HCV infection frequently evolves to chronicity and can progress to liver cirrhosis and hepatocellular carcinoma [Alter et al., 1989; Saito et al., 1990]. Coinfection with HCV and human immunodeficiency virus (HIV) is common in high-risk groups [McNair et al., 1992]. Epidemiological studies support the view that infection with HIV may facilitate vertical [Zanetti et al., 1995] and sexual [Eyster et al., 1991] transmission of HCV, possibly due to the high levels of HCV RNA detected in patients with HIV coinfection [Eyster et al., 1994; Ohto et al., 1994; Sherman et al., 1993]

The possible influence of HIV on the clinical course of HCV infection is still unclear. A rapid progression of liver disease was reported in three anti-HIV–positive patients with post-transfusion non-A, non-B hepatitis [Martin et al., 1989] and in multi-transfused haemophiliacs positive for anti-HCV and anti-HIV [Eyster et al., 1993]. Progression towards severe HCV-related liver disease in HIV-infected subjects might be explained by increased HCV replication [Cribier et al., 1995; Eyster et al., 1994; Martin et al., 1989; Telfer et al., 1994]. Alternatively, therapy with zidovudine might contribute to liver failure [Eyster et al., 1993; Gradon et al., 1992].

The HCV genome displays a marked sequence variability [for a review, see Simmonds, 1995]. Six viral genotypes have been identified so far, and sequence divergences were observed among different viral isolates in the same host [Martell et al., 1992; Weiner et al., 1991, 1992]. The infecting viral genotype [Tokita et al., 1994] and the degree of sequence heterogeneity [Honda et al., 1994; Kanazawa et al., 1994; Okada et al., 1992; Weiner et al., 1992; Zonaro et al., 1994] may influence the clinical profile of liver disease or the sensitivity to interferon therapy. A hypervariable region (HVR1) located at the N terminus of the E2/NS1 protein is highly divergent among different individuals. HVR1 contains a B-cell epitope [Kato et al., 1992, 1993; Weiner et al., 1993] that might be the target of neutralising antibodies. The heterogeneity of the HVR1 sequence is believed therefore to result from the immunogenic escape during persistent infection, as with the V3 loop in gp120 of HIV [Albert et al., 1990]. Although a recent study showed total conservation of the HVR1 sequence over a period of 2.5 years in an agammaglobulinaemic patient [Kumar et al., 1994], little is known on the possible influence of the concurrent HIV infection on the evolution of the HVR1 sequence.

We studied retrospectively a case of simultaneous infection with HIV and HCV through the determination of HCV and HIV viral titre and the genomic characterization of HCV HVR1 sequence over a 3.5-year period.

MATERIALS AND METHODS

Patient

On February 28, 1989, patient C. (female, aged 53) was assulted and stuck with a used syringe by a drug addict. On the same day her ALT levels were within normal range and the anti-HIV test was negative. Anti-HCV assays were not available.

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Address reprint requests to Elisabetta Cariani, III Laboratorio Analisi, Spedali Civili, p.le Spedali Civili 1, 25123 Brescia, Italy.

In May 1989, patient C. was admitted to the hospital with elevated ALT levels, malaise and jaundice. Examination of viral markers revealed the absence of markers of active HBV infection, of IgM against Epstein-Barr virus (EBV), and cytomegalovirus (CMV). Retrospective examination of a serum sample taken on admission to the hospital revealed absence of anti-HCV determined by second-generation enzyme-linked immunosorbent assay (ELISA). On the same serum sample, the second-generation recombinant immunoblot assay (RIBA) gave an indeterminate result, and serum HCV RNA could be determined by polymerase chain reaction (PCR). The anti-HIV ELISA was positive, with indeterminate Western Blot (FDA criteria), and detectable circulating viral RNA.

The examination of further serum samples, taken in July 1989, confirmed the occurrence of seroconversion to anti-HCV and anti-HIV. Fluctuating ALT levels, with persistence of HCV RNA and HIV RNA, were observed until 1993. In November 1991 a liver biopsy was performed which revealed aspects of acute hepatitis with bridging necrosis, evolving to chronicity. CD4+ cell counts were lower than normal values in May 1989 and, after an initial increase, remained stable at about 350–400/mm^3 until August 1992, when a sharp decrease accompanied by *Candida* oesophagitis was observed (AIDS defining condition CDC 1987). Treatment with zidovudine (250 mg twice daily) was undertaken in November 1989 but had to be stopped in February 1991 following the appearance of anaemia and leucopenia. The patient subsequently received interferon-α therapy from January to November 1992 at a dose of 6MU three times per week for 3 months and 9MU three times per week for 7 months. The treatment was suspended once again as a result of the appearance of symptomatic hypothyroidism.

In January 1993, patient C., suffering from decompensated cirrhosis with ascites, encephalopathy, and oesophageal varices, was admitted to the hospital. In July 1993 she died of pulmonary edema following an infectious pneumonia not linked to opportunistic infection. The autopsy revealed liver steatosis, micronodular cirrhosis, oesophageal varices, and ascites.

Serological Assays

Serum samples were tested for anti-HCV by second-generation assay (Abbott HCV EIA, Abbott Diagnostics, Abbott Park, IL) and recombinant immunoblot assay (Chiron RIBA HCV, Ortho Diagnostics, Raritan, NJ). The presence of anti-HIV was detected by ELISA (Abbott Recombinant HIV-1 EIA, Abbott Diagnostics) and Western blot (HIV-1 Western Blot, Diagnostic Biotechnology, Singapore).

The detection of HIV-1 p24 (core) antigen was performed by the Coulter ICD-Prep Kit, for the dissociation of HIV-1 antigen/antibody complexes in serum, and by the Coulter HIV-1 p24 Antigen Assay (Coulter Corporation, Hialeah, FL).

Amplification of HCV RNA

RNA was extracted from 200 μl of serum that had been stored at −80°C until used, with RNazol (Cinna/Biotecx Laboratories International, Friendswood, TX) following the manufacturer's instructions. The reverse transcription and amplification of the 5′ untranslated region of HCV (5′ UTR) by reverse transcription (RT)-PCR was performed as previously described [Puoti et al., 1992].

For the amplification of the E1/E2 region, cDNA synthesis and first step of nested PCR were performed with primers P and HC4, and 3 μl of the first reaction mix was amplified with internal primers CLK2 and CLK3 (nt 498-1259) as previously described [Ravaggi et al., 1994].

Typing of HCV Genome

Determination of HCV subtypes 1a and 1b was carried out by differential hybridization of E1 region (primers HE2–HE3) as described previously [Ravaggi et al., 1994]. Serum samples that could not be characterised by this method were subjected to the nucleotide sequence analysis of the E1 gene.

Quantification of HCV RNA by Competitive RT-PCR

The determination of the HCV RNA copy number was carried out by a competitive PCR-differential hybridisation assay, as previously described [Ravaggi et al., 1995].

Amplification of HIV Gag Region by RT-PCR

The detection of serum HIV RNA was undertaken by PCR amplification of a conserved 115-bp sequence of HIV-1 gag region [Ou et al., 1988]. For cDNA synthesis 50 pmol of antisense primer SK39 was used. For PCR, 50 pmol of sense primer (SK38) was added, and 40 cycles were performed as follows: denaturation for 1 min at 92°C, annealing of primers for 30 sec at 55°C, and extension for 1 min at 72°C. The specificity of the PCR product was assessed by hybridisation with the SK19 [Ou et al., 1988] probe with the DNA enzyme immunoassay (GENETI-K DEIA, Sorin Biomedica, Saluggia, Italy).

Quantification of HIV RNA by RT-PCR

The determination of the HIV RNA copy number was carried out by the Amplicor HIV Monitor test (Roche Molecular Systems, Somerville, NJ) following the manufacturer's instructions.

Cloning and Sequencing of the H2 HVR1 region

The PCR products of the E1–E2 region obtained with primers CLK2-CLK3 [Ravaggi et al., 1994] were cloned in the Bluescript plasmid vector (Stratagene, La Jolla, CA), and ten independent clones were derived from each isolate. The nucleotide sequence analysis was then performed on denatured double-stranded DNA using the Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, OH). The sequence data were analysed by a computer program PC/GENE (Intelligenetics, Geel, Belgium).

Statistical Analysis of Results

The demographic and epidemiological characteristics were compared by Student's t-test, the χ-square test, and Fischer's exact test. The correlation between the titres of HCV and HIV RNA (log copies/ml) and between HCV RNA titre and p24 antigenaemia was analysed by Spearman's rank-order correlation coefficient. A P value of .05 or less was considered significant.

RESULTS

A case of simultaneous infection with HIV and HCV was studied retrospectively over 3.5 years, from exposure to the emergence of AIDS and liver failure. During the first 2 months after the detection of ALT increase, HCV RNA titre changed in parallel with ALT levels, with a reciprocal time course of p24 antigenaemia and HIV RNA (Fig. 1). Decreasing ALT levels were detected 5 months after exposure, despite persistence of serum HCV RNA.

Treatment with zidovudine was accompanied by an initial increase in p24 antigen and then by declining levels of the circulating protein. HIV RNA decreased, HCV RNA titre was stable, and ALT values remained in the normal range, with minor elevation, for 1 year. An increase in HCV RNA titre was detected before an ALT peak in April 1991.

The liver biopsy, carried out in November 1991, revealed the presence of acute hepatitis with bridging necrosis. Treatment with interferon alpha (6MU 3 times per week) was accompanied by a decrease in ALT levels and by a decline of HCV RNA titre. An increased dose of interferon (9MU 3 times per week) was followed by an initial increase, then by a decline of the viral load. CD4+ cells decreased to less than 100/mm^3 (data not shown), with elevation of p24 antigenaemia and of HIV RNA. After the treatment was suspended, a sharp increase in HCV RNA titre was detected together with a decline of p24 antigenaemia.

Altogether, the results show a reciprocal time course of HCV and HIV viraemia. An inverse correlation was indeed observed between the titres of HCV RNA and HIV RNA (log copies/ml) evaluated on 7 samples available for analysis, ($r = -0.74$, $P = 0.05$), and between HCV RNA titre and p24 antigenaemia ($r = -0.58$, $P = 0.04$), evaluated on 12 samples (Fig. 1).

The HCV genotype was determined by differential hybridisation of E1 sequences in five independent serum samples collected throughout the follow-up period, detecting a mixed viral population of subtypes 1a and 1b in each specimen (data not shown). A partial sequence analysis of the amplified E1/E2 region (nt 1029–1257, aa 343–419) was performed on 11 serum samples (Fig. 1, bottom, and Fig. 2). In correspondence to the first ALT peak, only sequences belonging to subtype 1a could be detected, and the HVR1 sequence was completely homogeneous (variant HV1, Figs. 1, 2). The decrease in ALT and HCV RNA titre was accompanied by the emergence of a viral species belonging to subtype 1b (variant HV2). A third variant (HV3, subtype 1b) was detected in April 1991, when it emerged in correspondence to an ALT peak (Fig. 1). These results suggest that patient C. was simultaneously infected with HCV subtypes 1a and 1b. In the first period of infection, the HV1 variant, belonging to subtype 1a, appeared to take over from the HV2 variant (subtype 1b) in correspondence to HCV RNA peaks. Variant HV3 (subtype 1b) was also associated with decreased HCV RNA levels (Fig. 1, bottom).

The sequence heterogeneity of the HVR1 region within each variant was limited (Fig. 2). Two amino acid substitutions in the HVR1 of variant HV1 (F to V at residue 399 and N to D at residue 410) were identified in February 1991 and were detected in all samples analyzed thereafter. From their first detection, the HVR1 sequences of variants HV2 and HV3 were totally conserved over 2.5 and 1.5 years, respectively. It is unclear whether, in the early period of infection, variant HV3 was already present but replicating at a lower rate, or whether it derived from variant HV2. The presence of amino acid divergences between HV2 and HV3 located outside the HVR1 sequence and conserved in all isolates (Fig. 2) strongly supports the former hypothesis.

DISCUSSION

We studied retrospectively the natural course of mixed infection with HIV and HCV in a simultaneously infected subject. HCV infection was rapidly progressive towards liver cirrhosis, with evidence of portal hypertension within 4 years from exposure. The acute phase of HCV infection was followed by ALT normalisation despite persistence of serum HCV RNA. During the last 1.5 years of observation, ALT values were increased persistently with higher mean titre of HCV RNA.

The HIV RNA load showed a decrease during zidovudine treatment, then continued decreasing until the last period of observation, when a moderate increase was detected. The levels of p24 antigen initially increased during therapy; this was in accordance with previous studies which reported inconsistencies between the infectious virus and p24 antigen quantitation [Weber and Ariyoshi, 1992]. Despite the lack of correlation between p24 antigen and HIV RNA titre in single samples, the time course of both parameters was inversely related to HCV RNA levels, suggesting an interference between the replication of HCV and HIV. Since HCV replicative intermediates were detected in peripheral blood mononuclear cells of chronically infected subjects [Zignego et al., 1992] and replication of HCV was obtained in lymphoid cell culture [Shimizu et al., 1992], it is tempting to speculate that interference between HIV and HCV may occur in CD4+ cells. The naturally occurring or pharmacologically induced suppression of HIV replication might determine the reactivation of the HCV infection in CD4+ cells, thus causing a hepatitis relapse and interfering with the evolution of the liver disease. Consistent with this hypothesis, during zidovudine treatment the decline of HIV RNA titre was accompanied by a steady increase in HCV viraemia and by a subsequent flare-up of ALT levels. The findings suggest that the

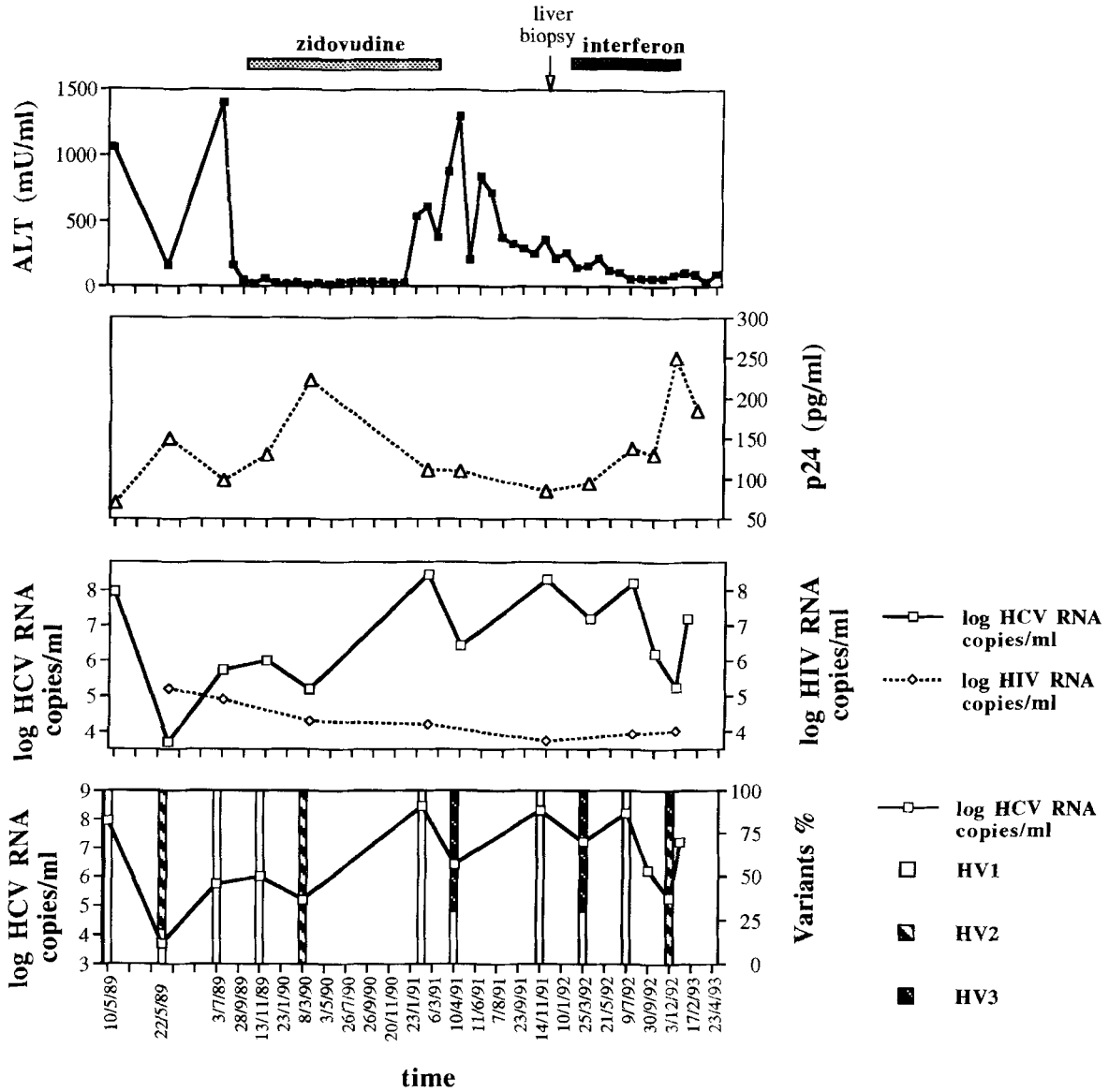

Fig. 1. Time course of clinical and laboratory parameters of patient C.

reactivation of hepatitis observed after zidovudine treatment was related to the increased HCV titre rather than to direct toxicity of the drug, as reported previously [Eyster et al., 1993]. Indeed no histological sign of toxic liver damage was detected in patient C. after zidovudine treatment and ALT flare-up.

Decreased ALT values and HCV RNA titre were detected during interferon therapy. The increase in p24 antigen and HIV RNA during treatment is in contrast with previous reports [DeWit et al., 1988]. This inconsistency could be explained by the different methods used for p24 antigen detection. Alternatively, the reactivation of HIV replication might be related to the suppression of HCV induced by interferon treatment.

A mixed infection with subtypes 1a and 1b was detected throughout. Previous studies detected higher prevalence of double HCV infections in anti-HIV-positive subjects [Ravaggi et al., 1994; Soriano et al., 1995], although the interplay between different viral types in the natural course of infection is unknown. In the present study, a viral variant belonging to subtype 1a was dominant in samples with high-titre replication. When HCV RNA titre decreased, two additional viral variants belonging to subtype 1b emerged, as a single or mixed viral population. These observations are suggestive of simultaneous infection with different viral strains, one of which (HV1) took over since the acute phase, possibly as a result of higher replicative potential. Whether this may be related to the different viral subtype or to the multiplicity of each strain in the original inoculum remains open to speculation.

The sequence analysis of the E2 hypervariable region (HVR1) detected limited evolution in the HVR1 of variant HV1. Mutated HV1 remained as a dominant strain

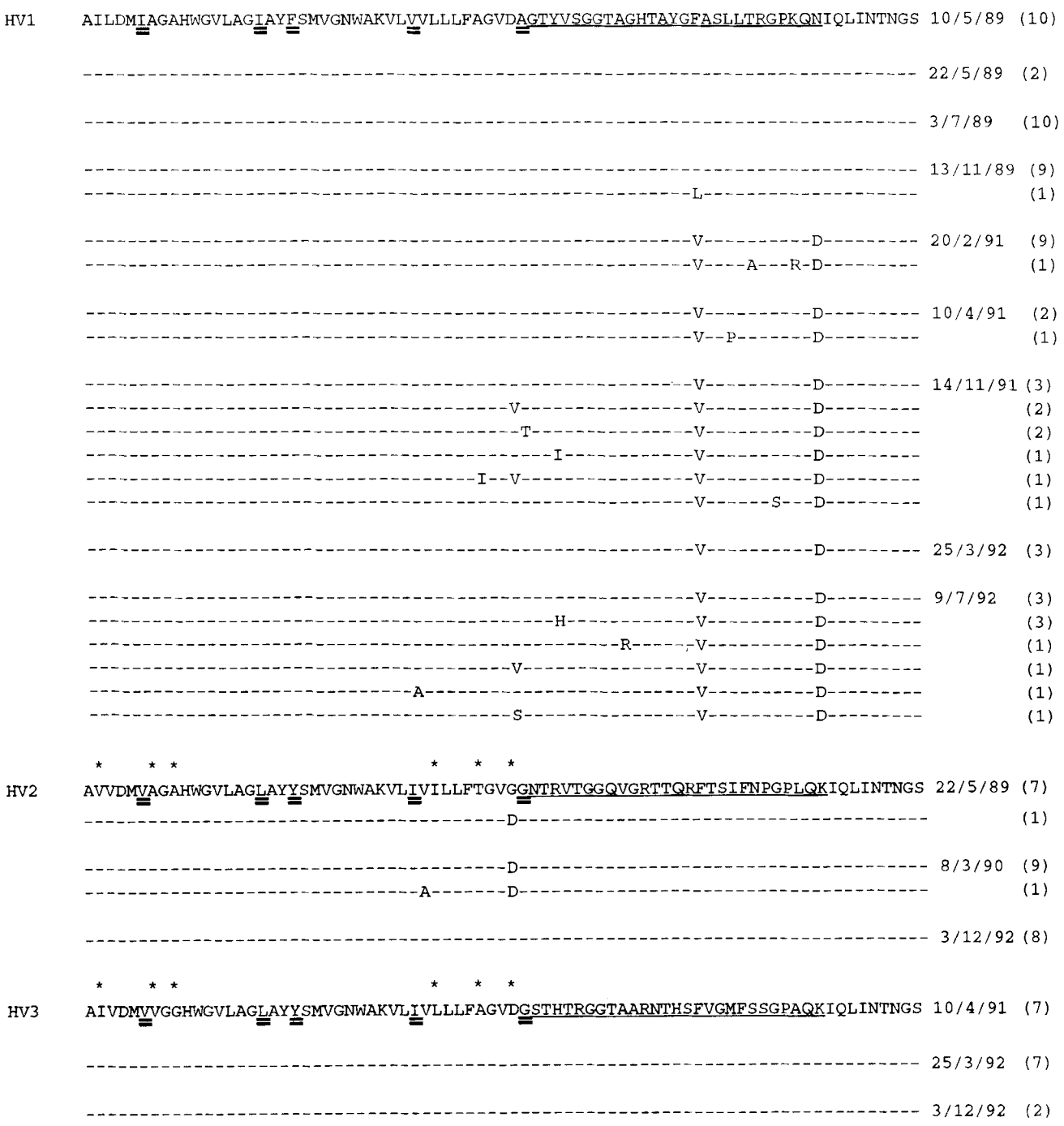

Fig. 2. Deduced amino acid sequence of the E1–E2 region (residues 343-419) derived from serum samples of patient C. The hypervariable region 1 (HVR1) is underlined. Double underline indicates residues specific for subtypes 1a (variant HV1) and 1b (variants HV2 and HV3). *: amino acid divergences between HV2 and HV3 located outside HVR1. The number of clones with each sequence is indicated in parentheses.

in all subsequent samples with peak HCV RNA titre, and the HVR1 sequence of variants HV2 and HV3 was completely conserved during the observation period. These results suggest that a higher rate of replication during the first phase of infection exposed variant HV1 to the host immune pressure, which may subsequently have brought about the selection of HVR1 mutations. By contrast, variants emerging in the more advanced stages of HIV infection underwent lower evolutionary pressure and, as a consequence, no HVR1 mutation was selected. Overall consistent with recent findings [Sherman et al., 1996], the host immune response seemed unable to eliminate HVR1 variants present in the former stages of infection, as often occurs in the

immune-competent host [Kato et al., 1993; Weiner et al., 1992].

Altogether the longitudinal follow-up of a patient with double HCV infection and rapidly progressive clinical course indicated the presence of interference between HIV and HCV and between HCV subtypes. In addition, an ineffective immune response against HCV was suggested by the persistence and sequence conservation of the HVR1 variants isolated during follow-up. The role played by viral interferences and by impaired immune response on the clinical evolution of HCV infection will be better clarified by prospective studies on large populations of patients coinfected with HIV.

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